THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 13, pp. 11254–11264, April 1, 2011

Differential Regulation of Gene Expression by Protein Kinase C Isozymes as Determined by Genome-wide Expression Analysis*[S]

Received for publication, October 13, 2010, and in revised form, January 18, 2011 Published, JBC Papers in Press, January 20, 2011, DOI 10.1074/jbc.M110.194332

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Protein kinase C (PKC) isozymes are key signal transducers involved in normal physiology and disease and have been widely implicated in cancer progression. Despite our extensive knowledge of the signaling pathways regulated by PKC isozymes and their effectors, there is essentially no information on how individual members of the PKC family regulate gene transcription. Here, we report the first PKC isozyme-specific analysis of global gene expression by microarray using RNAi depletion of diacylglycerol/phorbol ester-regulated PKCs. A thorough analysis of this microarray data revealed unique patterns of gene expression controlled by PKCα, PKCδ, and PKCε, which are remarkably different in cells growing in serum or in response to phorbol ester stimulation. PKCδ is the most relevant isoform in controlling the induction of genes by phorbol ester stimulation, whereas PKCε predominantly regulates gene expression in serum. We also established that two PKCδ-regulated genes, *FOSL1* and *BCL2A1*, mediate the apoptotic effect of phorbol esters or the chemotherapeutic agent etoposide in prostate cancer cells. Our studies offer a unique opportunity for establishing novel transcriptional effectors for PKC isozymes and may have significant functional and therapeutic implications.

Protein kinase C (PKC) isozymes are important signal transducers involved in normal physiology and numerous diseases, including cardiovascular, neurological, and proliferative dysfunctions. In addition, PKCs are well established players in oncogenesis through modulation of multiple signaling pathways involved in differentiation, survival, and apoptosis. This family of serine-threonine kinases comprises three groups of isozymes with unique biochemical properties: classical/conventional or calcium-dependent PKCs (cPKCs)[3] α, β, and γ; novel or calcium-independent PKCs (nPKCs) δ, ε, η, and θ; and atypical PKCs (aPKCs) ζ and λ. Only members of the cPKC and the nPKC classes respond to the receptor-generated lipid second messenger diacylglycerol (DAG) or DAG mimetics, such as the phorbol esters, an event that occurs through specific binding to the PKC C1 domains (1, 2). Despite their high homology and similar substrate specificity *in vitro*, PKC isozymes possess striking functional selectivity in cells due to their distinctive intracellular localization and differential access to substrates (1). Although in many cases PKCs have overlapping effects, there is extensive evidence for unique biological responses mediated by individual PKC isozymes. This is well illustrated by members of the novel PKC family, namely PKCδ and PKCε, which exert opposite effects particularly in the context of mitogenesis and survival (1, 3, 4). PKCδ has been indeed implicated in growth arrest via $p21^{cip1}$ up-regulation and pRb dephosphorylation, and it mediates apoptotic cell death in response to various stimuli through the activation of both the intrinsic and extrinsic apoptotic cascades (5, 6). On the other hand, PKCε mostly drives mitogenic responses via the Raf-MEK-ERK cascade and plays important roles in cell survival (7, 8). Androgen-responsive prostate cancer cells exemplify such divergence of PKC isozyme function. For example, in LNCaP cells, PKCδ is an essential mediator of the apoptotic responses to phorbol 12-myristate 13-acetate (PMA/TPA) or chemotherapeutic drugs such as etoposide, whereas PKCε, which is up-regulated in human prostate cancer, significantly contributes to LNCaP cell survival and mediates transition to androgen-independence (6, 9–13). Very recent studies revealed that PKCδ and PKCε have opposite roles in the secretion of TNFα, a death factor implicated in prostate cancer cell apoptosis, and distinctively modulate cell death induced by this cytokine in prostate cancer cells (6, 12, 14, 15). PKCα has also been implicated in apoptotic signaling in LNCaP cells through its ability to negatively modulate the Akt survival pathway (10).

It has long been known that phorbol esters cause profound changes in gene expression, although the relative contribution of transcriptional events to PKC-mediated responses is far from being understood. Early studies identified TPA/PMA-responsive elements in gene promoters, and it was subsequently established that phorbol esters modulate gene expression through multiple pathways, including ERK, JNK/AP1, NF-κB, and JAK/STAT cascades (16–21). Despite a few reports of genes differentially regulated by PKC isozymes, such as *GM-CSF*, *NOX4*, and *IL-8* (22–24), there is essentially no information regarding the ability of individual members of the PKC family to regulate gene expression in a global manner.

* This work was supported, in whole or in part, by National Institutes of Health Grant CA89202 (to M. G. K.).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S6 and Figs. S1–S3.

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[3] The abbreviations used are: cPKC, nPKC, and aPKC, classical/conventional or calcium-dependent, novel or calcium-independent, and atypical PKC, respectively; DAG, diacylglycerol; GO, gene ontology; GSEA, gene set enrichment analysis; FDR, false discovery rate; PMA, phorbol 12-myristate 13-acetate; MSigDB, Molecular Signature Database; TPA, 2-*O*-tetradecanoylphorbol-13-acetate.

Here we carried out the first PKC isozyme-specific genome-wide analysis using LNCaP androgen-dependent prostate cancer cells. Not only did we establish unique patterns of gene expression controlled by individual PKCs, but we also identified novel PKCδ-specific regulated genes implicated in LNCaP cell death. Moreover, our studies defined a differential utilization of PKC isozymes in gene expression regulation depending on the stimuli.

EXPERIMENTAL PROCEDURES

Reagents, Antibodies, and RNAi Sequences—PMA was purchased from LC Laboratories (Woburn, MA). 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Sigma. Etoposide was purchased from EMD (San Diego, CA). The following antibodies were used: anti-PKCα (Millipore), anti-PKCδ (Cell Signaling, Danvers, MA), anti-PKCϵ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-β-actin (Sigma). For RNAi experiments, the following target sequences were used: Silencer negative control 1 (AM4615) and Silencer negative control 2 (AM4644) (Applied Biosystems/Ambion, Austin, TX); PKC α1, CCAUCCGCUCCACACUAAA; PKC α2, GAACAACAAGGAAUGACUU; PKC δ1, CCAUGAGUUUAUCGCCACC; PKC δ2, CAGCACAGAGCGUGGGAAA; PKC ϵ1, GUGGAGACCUCAUGUUUCA; and PKC ϵ2, GACGUGGACUGCACAAUGA (Dharmacon, Lafayette, CO). To knock down *FOSL1* (FOS-like antigen 1), *BCL2A1* (BCL2-related protein A1), *SERPINB2* (serpin peptidase inhibitor, clade B (ovalbumin), member 2), or *TRAF1*, we used ON-TARGETplus SMARTpool RNAi from Dharmacon (*FOSL1* L-004341-00, *BCL2A1* L-003306-00, *SERPINB2* L-010859-00, and *TRAF1* L-017438-00). Additional siRNAs for *BCL2A1* and *FOSL1* were as follows: *BCL2A1* 1, GCAGUGCGUCCUACAGAUA; *BCL2A1* 2, UAUCUCUCCUGAAGCAAUA; *FOSL1* 1, GCUCAUCGCAAGAGUAGCA; and *FOSL1* 2, GAGCUGCAGUGGAUGGUAC.

General Methods—Cell culture, Western blotting, and RNAi transfection were carried out as described previously (12). Apoptosis assays and qPCR were described previously (6). Real-time PCR analysis using Taqman universal PCR master mix was performed on a 7300 real-time PCR system (Applied Biosystems). Taqman gene expression assays were purchased from Applied Biosystems.

RNA Isolation and cDNA Synthesis—Cells (6×10^5) were treated with either vehicle (ethanol) or PMA (1 h, 100 nM), and total RNA was isolated at different times post-treatment (4, 8, 12, and 24 h). Total RNA isolated with TRIzol (Invitrogen) was further purified using a Qiagen RNeasy kit. One μg of RNA was reverse transcribed to cDNA using random hexamers as primers and the Taqman reverse transcription reagents kit (Applied Biosystems).

DNA Microarray and Analysis of Data—Experiments were performed in triplicate using the Affymetrix GeneChip® human genome U133A 2.0 array at the University of Pennsylvania Microarray Facility. All protocols were conducted as described in the NuGEN Ovation and the Affymetrix GeneChip Expression Analysis technical manuals.

Samples were hybridized to the array, and cell intensity files (CEL files) were extracted from raw data files (DTT files). For background correction, normalization, and probe summarization, the GeneChip robust multiarray averaging algorithm was applied using the program ArrayAssist Lite version 3.4 (Stratagene, La Jolla, CA). For statistical analysis, data were evaluated using the Partek Genomics suite (Partek Inc., St Louis, MO). The $\log_2$ (treatment/control) ratio for each probe set was calculated, and p values from one-way analysis of variance followed by false recovery rate (FDR) correction were calculated according to Benjamini and Hochberg (49). Probes were considered for further evaluation if their FDR-corrected p value for the analysis of variance was $p \leq 0.001$. For single-gene analysis, probe sets were filtered by expression change being at least ±2-fold in any one of the conditions analyzed. For gene set analysis (see below), data from all probe sets that were statistically significant were used.

Gene Set Enrichment Analysis (GSEA)—A computational approach to evaluate the microarray data at the level of gene sets was undertaken by applying GSEA (25). For each PKC isozyme, samples from two classes were compared: PKC-expressing *versus* PKC-depleted ($PKC^+ > PKC^-$). Defined gene sets from the Molecular Signature Database version 3.0 (MSigDB) were used to evaluate whether statistically significant differences existed between the two groups. The gene ontology (GO) catalogue (available on the Gene Ontology Web site) was downloaded from the MSigDB and used for $PKC^+ > PKC^-$ comparisons. An independent GSEA was carried out to

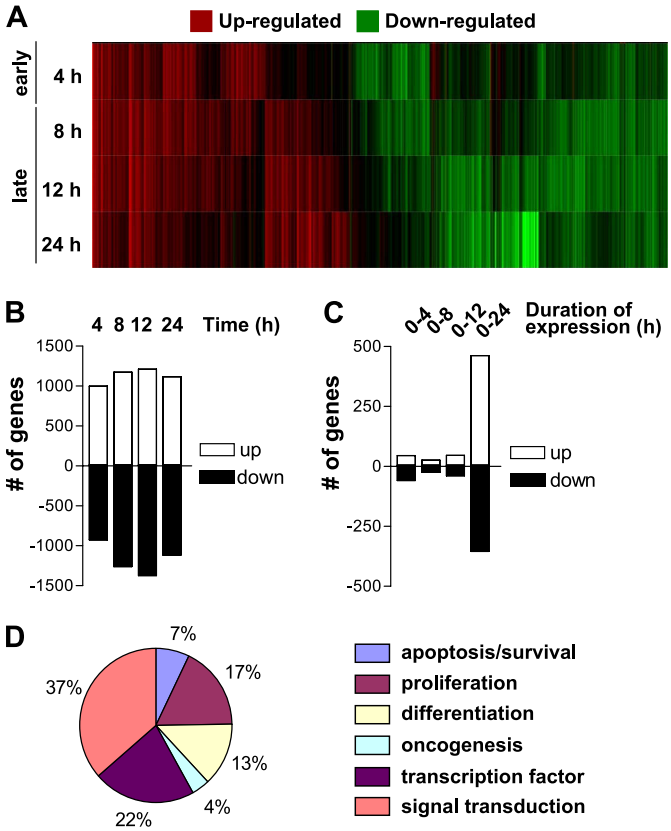


FIGURE 1. **PKC activation by PMA induces changes in gene expression.** *A*, heat map of changes in gene expression induced by PMA in LNCaP cells at different times. *B*, time-dependent distribution of genes regulated by PMA at different time points. *C*, duration of expression changes of early genes. *D*, classification of early regulated genes according to relevant gene ontology biological processes.

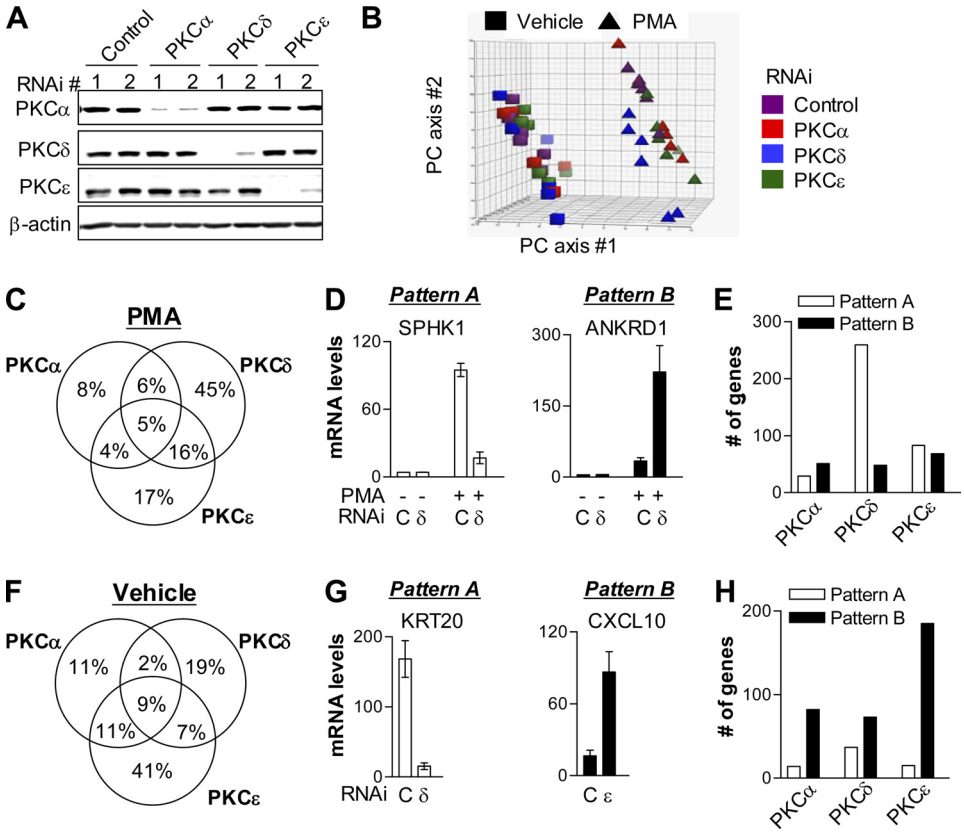


FIGURE 2. **Differential contribution of PKC isozymes to gene expression.** *A*, Western blot of LNCaP cells transfected with two different RNAi duplexes (*#1* or *#2*) for each PKC isozyme. A representative experiment is shown ($n = 3$). *B*, principal component (*PC*) analysis of the microarray data comparing vehicle and PMA. *C*, Venn diagram showing the contribution of PKC isozymes to gene regulation by PMA. *D*, example of genes regulated by PMA via PKCδ in either positive (*Pattern A*) or negative (*Pattern B*) manners. *E*, total number of PMA-regulated genes by each PKC isozyme. *F*, Venn diagram showing the contribution of PKC isozymes to basal gene regulation. *G*, example of genes regulated by PKCs in either positive (Pattern A) or negative (Pattern B) manner in LNCaP cells growing in serum. *H*, total number of genes regulated by PKC isozymes in basal conditions. *Error bars*, S.E.

identify apoptosis/survival gene set enrichment in PKCδ$^+$ and PKCε$^+$ samples. In this case, we browsed the MSigDB for gene sets containing the "Apoptosis" or "Survival" terms and exported them as a user-defined collection (supplemental Table S1). A total of 1,000 permutations were run to obtain the enrichment score, associated *p* value, and FDR, as described previously (25). Where indicated, a post-GSEA leading edge analysis (25) was performed with the top-scoring enriched gene sets for each PKC isozyme to obtain the common core genes regulated by each PKC.

Oncomine Analysis—The Oncomine database (available on the World Wide Web) was searched for PKCδ-regulated genes. The data sets containing expression data for each gene were further filtered to display down-regulation in prostate cancer *versus* normal prostate tissue with $p < 0.05$. If more than one data set passed the filters, we performed a meta-analysis to obtain a *p* value. Box plots were generated for the expression of PKCδ-regulated genes grouped by cancer and normal types.

In order to investigate whether the underexpression of PKCδ-regulated genes was associated with specific biologically relevant aspects of prostate cancer, we ran an "Oncomine Concept Analysis." Association between down-regulation of PKCδ target genes and either androgen ablation-resistant prostate cancer or prostate cancer samples with Gleason scores compared with normal prostate was analyzed. *p* values, FDR values, and odds rates were obtained for each association.

Statistical Analysis—For microarray data, statistical analysis was performed using the Partek Genomics suite (Partek Inc.). For all other data sets, GraphPad Prism version 3.0 was used. Two means were compared by unpaired Student's *t* test. $p < 0.05$ was considered as significant.

RESULTS

Genome-wide Analysis in Response to PKC Activation—To begin assessing global changes in LNCaP gene expression, we carried out a time-dependent microarray analysis in response to phorbol ester treatment. LNCaP cells were treated with PMA (100 nM, 1 h), and at different times after treatment (4, 8, 12, and 24 h), RNA from three replicates for each group was extracted and reverse transcribed, followed by microarray analysis using Affymetrix GeneChip® human genome arrays. Using a 2-fold change relative to vehicle-treated cells as a cut-off, we found that 4,949 genes displayed statistically significant changes in expression in response to PMA, with a similar proportion of up- and down-regulated genes (Fig. 1, *A* and *B*). The top 50 genes are listed in supplemental Table S2 (experiment 1 (*Exp#1*) in the table). PMA-regulated genes with their corresponding time courses of expression are presented in supplemental Table S3. A more detailed analysis revealed that 39% of the genes change their expression at 4 h (1,013 up-regulated and 931 down-regulated genes), whereas secondary waves of gene expression manifest at later times (Fig. 1*B*). Although a small number (104

early genes) return to basal levels at 8 h, most changes are sustained, and 41% of the genes remain either up- or down-regulated for at least 24 h (Fig. 1*C*). Categorization of early PMA-regulated genes according to six gene ontology biological processes led to the functional classification of 589 genes (Fig. 1*D* and supplemental Table S4). Among the most characteristic genes with the larger inductions (>100-fold), we found multiple cytokines, including *CCL2*, *IL-8*, and *TNFα*. A prominent secretion of these cytokines from LNCaP cells was observed in response to PMA, as determined using a cytokine array and ELISA (supplemental Fig. S1) (6). A marked up regulation of metalloproteinase genes (*MMP1*, *MMP2*, and *MMP10*) was also evident, consistent with the well established role of PKCs in invasiveness (1).

Unique and Overlapping Patterns of Gene Regulation by PKC Isozymes; PKCδ as a Major Regulator of Gene Induction—A fundamental issue that remains to be addressed is whether individual PKC isozymes have distinctive roles in the control of gene expression. The well established differential effects of PKCs in survival and apoptosis in prostate cancer cells prompted us to examine whether each of the three PMA-responsive PKCs present in LNCaP cells (PKCα, PKCδ, and PKCε) (12) could potentially regulate different subsets of genes. To this end, we carried out a genome-wide expression analysis in LNCaP cells subject to RNAi depletion for distinct PKC isozymes. Various siRNA duplexes at different concentrations were tested (data not shown), and the two most effective and specific for each PKC were selected for these studies in order to minimize the chances of misinterpretation of data due to "off-target" effects. Two different non-targeting siRNA sequences that did not affect PKC levels were employed as controls. As shown in Fig. 2*A*, we achieved nearly complete depletion with all RNAi duplexes selected, and importantly, knockdown was PKC isozyme-specific.

PKCα-, PKCδ-, and PKCε-depleted and control LNCaP cells (two different RNAi sequences, three replicates for each) were treated with either vehicle (ethanol) or PMA (100 nM, 1 h). To focus only on early genes, samples were collected at 4 h post-PMA treatment. Gene expression profiles for the 48 resulting samples were obtained using Affymetrix chips. Only those genes that displayed statistically significant changes in all replicates and showed no significant differences between the two different siRNA duplexes were considered for further analysis. There was a remarkable reproducibility for the early PMA-up-regulated genes between this array and the array shown in Fig. 1 (96% concordance) (supplemental Table S2). A rough estimation of the variability in gene expression across the whole genome was obtained by principal component analysis. In addition to the profound differences between PMA- and vehicle-treated LNCaP cells, PKCδ-depleted samples were differentially positioned, suggestive of a high number of genes distinctively regulated by this isoform in response to PMA (Fig. 2*B*).

As a first approach to gain insight into the biological responses controlled by PKC-regulated genes, we evaluated the expression profiles from PKC-expressing *versus* PKC-depleted samples ($PKC^+ > PKC^-$) using GSEA (25). In all cases, we searched for GO gene sets available through the MSigDB. We first applied GSEA to identify GO gene sets correlated with PKCδ-expressing status and identified 22 gene sets with FDR of <0.25 (Table 1, *top*). Among the top-scoring enriched gene sets, we found three related to apoptosis, which fits with the well established role of PKCδ in PMA-induced cell death in LNCaP cells (6, 10). The presence of other GO gene sets enriched in PKCδ-expressing cells suggests other potential functions for PKCδ in these cells.

TABLE 1

Gene set enrichment analysis for correlation of gene ontology gene sets with PKC isozymes

Genome-wide expression profiles obtained by microarray were analyzed by GSEA. A collection of GO gene sets from MSigDB were used to test for correlation with the expression status of each individual PKC isozyme ($PKC^+ > PKC^-$). The top scoring gene sets are presented with their associated *p* values, FDR, and enrichment signal. Gene sets related to apoptosis are highlighted.

GO-gene set correlated to PKCδ	p-value	FDR	Signal
EPIDERMIS_DEVELOPMENT	$< 1 \times 10^{-6}$	0.001042	23%
EXTRACELLULAR_SPACE	$< 1 \times 10^{-6}$	0.003221	16%
ECTODERM_DEVELOPMENT	$< 1 \times 10^{-6}$	0.02553	19%
EXTRACELLULAR_REGION_PART	$< 1 \times 10^{-6}$	0.030472	14%
LOCOMOTORY_BEHAVIOR	$< 1 \times 10^{-6}$	0.044759	16%
STRUCTURAL_MOLECULE_ACTIVITY	$< 1 \times 10^{-6}$	0.039434	6%
REGULATION_OF_PROTEIN_MODIFICATION_PROCESS	$< 1 \times 10^{-6}$	0.060502	11%
REGULATION_OF_PROGRAMMED_CELL_DEATH	$< 1 \times 10^{-6}$	0.05415	24%
REGULATION_OF_APOPTOSIS	$< 1 \times 10^{-6}$	0.054351	24%
BEHAVIOR	$< 1 \times 10^{-6}$	0.057542	13%
EXTRACELLULAR_REGION	$< 1 \times 10^{-6}$	0.053086	12%
MULTI_ORGANISM_PROCESS	0.001387	0.192975	20%
CARBOXYLESTERASE_ACTIVITY	0.01675	0.199141	20%
G_PROTEIN_COUPLED_RECEPTOR_BINDING	0.001672	0.187835	19%
TISSUE_DEVELOPMENT	0.002736	0.183064	16%
CYTOKINE_ACTIVITY	0.005806	0.174046	17%
PROTEASE_INHIBITOR_ACTIVITY	0.018644	0.193724	22%
APOPTOSIS	0.002639	0.192764	23%
DIGESTION	0.006678	0.227645	29%
RESPONSE_TO_OTHER_ORGANISM	0.007899	0.21803	29%
RESPONSE_TO_VIRUS	0.009788	0.215501	27%
SERINE_TYPE_ENDOPEPTIDASE_INHIBITOR_ACTIVITY	0.015845	0.210208	21%

GO-gene set correlated to PKCε	p-value	FDR	Signal
METALLOENDOPEPTIDASE_ACTIVITY	0.01	1	37%
CHROMATIN	0.01	1	31%
DNA_HELICASE_ACTIVITY	0.03	1	15%
MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS	0.02	1	39%
MITOCHONDRIAL_MEMBRANE	0.01	1	89%
COVALENT_CHROMATIN_MODIFICATION	0.04	1	32%
GENERAL_RNA_POLYMERASE_II_TRANSCRIPTION_FACTOR_ACTIVITY	0.04	1	30%
MITOCHONDRIAL_INNER_MEMBRANE	0.02	1	107%
RESPONSE_TO_UV	0.04	1	11%
MITOCHONDRIAL_MEMBRANE_PART	0.02	0.98384583	113%
EXTRACELLULAR_STRUCTURE_ORGANIZATION_AND_BIOGENESIS	0.06	1	31%
CYTOPLASMIC_VESICLE_MEMBRANE	0.05	1	59%
VESICLE_MEMBRANE	0.07	1	54%
METALLOPEPTIDASE_ACTIVITY	0.05	0.97740865	29%
EXOCYTOSIS	0.07	0.9822864	30%
CYTOPLASMIC_VESICLE_PART	0.06	0.9488981	59%
CHROMATIN_MODIFICATION	0.04	0.95961183	21%
MITOCHONDRIAL_RESPIRATORY_CHAIN	0.07	0.9081712	121%
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	0.03	0.98563486	114%

GO-gene set correlated to PKCα	p-value	FDR	Signal
EXTRACELLULAR_STRUCTURE_ORGANIZATION_AND_BIOGENESIS	0.010246	1	30%
EXOCYTOSIS	0.009506	1	17%
SYNAPSE	0.019194	1	20%
SECONDARY_ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	0.030741	1	30%
DOUBLE_STRAND_BREAK_REPAIR	0.030303	1	28%
SYMPORTER_ACTIVITY	0.048825	1	39%
CALCIUM_CHANNEL_ACTIVITY	0.047619	1	26%
CARBOHYDRATE_BINDING	0.030822	1	12%
ANION_TRANSPORT	0.037975	1	6%
ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	0.01495	1	21%
REGULATION_OF_GENE_EXPRESSION_EPIGENETIC	0.04	1	20%
DEVELOPMENT_OF_PRIMARY_SEXUAL_CHARACTERISTICS	0.055357	1	6%
OXIDOREDUCTASE_ACTIVITY_GO_0016616	0.0434	1	12%
PROTEIN_AMINO_ACID_DEPHOSPHORYLATION	0.050251	1	30%
GENERATION_OF_A_SIGNAL_INVOLVED_IN_CELL_CELL_SIGNALING	0.076172	1	20%
RESPONSE_TO_RADIATION	0.051971	1	21%
PHOSPHOPROTEIN_PHOSPHATASE_ACTIVITY	0.036728	1	25%
ONE_CARBON_COMPOUND_METABOLIC_PROCESS	0.090909	1	28%
PATTERN_BINDING	0.093066	1	19%
RESPONSE_TO_ABIOTIC_STIMULUS	0.038035	1	11%
POSITIVE_REGULATION_OF_CASPASE_ACTIVITY	0.092937	1	32%
REGULATION_OF_HYDROLASE_ACTIVITY	0.06136	1	30%
PROTEIN_DOMAIN_SPECIFIC_BINDING	0.043845	1	26%
CHROMATIN	0.080935	1	24%
NEGATIVE_REGULATION_OF_MAP_KINASE_ACTIVITY	0.117647	1	36%

TABLE 2

Genes specifically regulated by only one PKC isozyme

Specificity was defined as >50% change in PMA-mediated induction upon depletion of one PKC isozyme with <25% change upon depletion of the other two PKCs. PKCα showed no specific regulation of gene expression using these specificity criteria. Mean expression values are presented for each RNAi as -fold change (PMA/vehicle). Pattern A, gene induction by PMA is reduced by RNAi; Pattern B, gene induction by PMA is augmented by RNAi.

PKCδ specific genes, *Pattern A*

	Fold change (PMA vs. vehicle)			
Gene/RNAi	CTRL	PKCα	PKCδ	PKCε
CCL20	432	445.4	205.8	371.2
LIPG	412.1	380.9	138.7	348
C3orf52	352.2	349.8	140.4	302.4
SERPINB2	347.6	277.5	69.1	280.9
MMP12	289.5	265.6	100.9	285.6
KIAA1199	279.8	268.4	84.5	222.7
BCL2A1	231.7	261.6	41.1	231
LBH	229.9	181.2	66.1	180.4
FOSL1	177.5	197.1	58.9	166.6
KHDC1	173.5	185.2	59.7	150.4
IL2RG	150.2	132.2	58.2	142.7
NAV2	141.7	124.8	57.3	137.4
C6orf54	140.3	149	64.4	126.3
GEM	139.1	104.5	25.2	154.5
KIAA1462	134.6	138.7	42.1	132
KLF6	118.7	105.6	49.7	89.6
ZEB1	111.1	96.1	52.9	98.2
ALOXE3	80.6	91.5	39.8	82.7
ARID3B	69.8	54.3	22.9	59.3
GPRC5A	65.4	78.7	11.7	52.1
TRAF1	52.9	58.8	15.3	46.3
LIF	49.6	51.3	17.6	51.6
LAMB3	48.2	57.5	23.6	50.7
PP1R15A	44.8	37.3	13	40
FBN1	26.8	26.1	10.5	30.5
MAST4	24.1	23.5	6	19.1
SPHK1	22.7	28	3.3	24.7
CAMSAP1	19.6	17.2	9.8	17.8
ICOSLG	19.6	18.9	9.3	18
CCRN4L	18.3	15.3	6.7	18.3
LDLR	18.1	13.7	8.3	15.3
ETS2	17.7	15.9	5.3	16.1
GPR87	16	12.7	3.7	15.7
CD4	11.4	9.6	3.3	9.4
PDLIM7	10.7	8.6	5.2	10.3
FEM1B	9.9	9.7	3.9	8.5
UGCG	7	5.4	3.5	5.4
RYBP	6.9	6.2	3.3	5.9
LYST	6.7	5.8	3.3	5.4
ITPR3	6.5	6.5	3.2	5.2
USP36	5.9	6.5	2.5	5.2
OAS1	5.8	4.9	2.6	4.4
APOBEC3A	5.4	6.3	1.5	5.9
SLC22A1	5.1	6.1	1.6	3.9
PLAGL2	5	4.9	2.3	4.3
CDKN2A	4.5	4.3	1.9	3.5
DENND3	4.5	5.6	1.3	3.4
IL1RN	3.8	3.8	1	3.4
TTC9	3.7	4.4	1.8	4.1
KIAA0999	3.7	3	1.6	3.1
CTSB	3.4	3.2	1.4	2.6
TRPC1	3.4	2.9	1.2	3.7
MYO9B	3.2	3.1	1.2	2.7
SOCS1	3.1	2.7	1.3	3.4
TPBG	3	3.8	1.3	3.5
SLC15A1	2.8	2.4	1.3	3.3
TNFRSF11B	2.2	1.9	1	1.8

TABLE 2—*continued*

PKCδ specific genes, *Pattern B*

	Fold change (PMA vs. vehicle)			
Gene/RNAi	CTRL	PKCα	PKCδ	PKCε
MRPS11	0.23813	0.32277	2.0224	0.27046
ERCC6L	0.17664	0.21482	0.4559	0.25448
RICS	0.14514	0.19946	0.46645	0.14664
FZD1	0.07841	0.08904	0.29548	0.10152
SETBP1	0.06579	0.07235	0.2704	0.0706
HJURP	0.04864	0.08767	0.2227	0.07525
ADAM7	0.04072	0.08144	0.29845	0.0394
PALMD	0.00998	0.01611	0.12849	0.00604
EPOR	0.00346	0.00736	0.10601	0.00929
PPP1R3D	0.0026	0.00127	0.05709	0.00123
KIF2C	0.00152	0.00281	0.0457	0.00494
GRHL2	0.00026	0.00015	0.01877	0.00137
TRIM48	9.2×10^{-7}	3.8×10^{-7}	0.00888	0.00002
SLITRK3	6.9×10^{-7}	1.1×10^{-6}	0.008	3.3×10^{-6}
SMAD6	6×10^{-7}	4.8×10^{-6}	0.00307	4.4×10^{-6}
C8orf51	3.8×10^{-7}	4.7×10^{-8}	0.00278	2.1×10^{-7}
OSR2	10×10^{-7}	3.1×10^{-7}	0.04022	7×10^{-7}
SPRY1	8.1×10^{-19}	5.6×10^{-17}	0.00001	9×10^{-19}

PKCε specific genes, *Pattern A*

	Fold change (PMA vs. vehicle)			
Gene/RNAi	CTRL	PKCα	PKCδ	PKCε
BIRC3	171.1	147.8	203.2	75.6
CYR61	157.8	188.3	125.9	78.7
RSG2	23.6	18	24.1	9

PKCε specific genes, *Pattern B*

	Fold change (PMA vs. vehicle)			
Gene/RNAi	CTRL	PKCα	PKCδ	PKCε
AMIGO2	0.154	0.203	0.235	2

Next, we ran a GSEA for PKCε-expressing *versus* PKCε-depleted profiles ($PKC\epsilon^{+} > PKC\epsilon^{-}$) (Table 1, middle). 15 gene sets were significantly enriched at nominal p value of <0.05, including three gene sets related to mitochondrial structure/function with >50% of enrichment signal. Interestingly, we recently demonstrated that PKCε translocates to mitochondria in order to exert some of its prosurvival functions (12). However, unlike PKCδ, none of the GO gene set for PKCε passed the threshold FDR < 0.25. Similarly, despite the identification of 19 gene sets for PKCα encompassing various functions enriched at nominal p value < 0.05, GSEA did not retrieve any significant (FDR < 0.25) GO gene sets correlated with PKCα-expressing status (Table 1, bottom). Therefore, results from PKCα or PKCε should be interpreted with caution due to the high chance of false correlations.

Altogether, we conclude that there is essentially no overlap between GO gene sets regulated by PKCα, PKCδ, and PKCε, which strongly argues for functional diversity in the regulation of gene expression by PKC isozymes. A second conclusion is that, unlike PKCα and PKCε, PKCδ seems to have a prominent role in controlling cellular functions through coordinated regulation of genes.

Single-gene Analysis Defines PKC Isozyme-specific Induction of Genes in Response to PMA—In order to identify those genes specifically regulated by each PKC isozyme, we carried out

TABLE 3

GSEA results for apoptosis *versus* survival gene set correlation in PKCδ and PKCε samples

Genome-wide expression profiles obtained by microarray were analyzed by GSEA. A collection of apoptosis or survival gene sets from MSigDB were used to test for correlation with the expression status of each individual PKC isozyme. The top scoring gene sets are presented with their associated enrichment scores (ES), *p* values, FDR, and enrichment signal. Note that for PKCε, the apoptotic gene sets did not pass the threshold of significance (FDR < 0.25 and $p < 0.05$). Survival gene sets are positively correlated to PKCε (ES > 0) and inversely correlated to PKCδ (ES < 0).

	$PKC\delta^+ > PKC\delta^-$			$PKC\varepsilon^+ > PKC\varepsilon^-$		
Apoptosis-related gene sets	**ES**	**p-value**	**FDR**	**ES**	**p-value**	**FDR**
NEGATIVE_REGULATION_OF_APOPTOSIS	-0.5939	$< 1 \times 10^{-6}$	0.123529	0.288156	0.587684	0.866665
CONCANNON_APOPTOSIS_BY_EPOXOMICIN_UP	-0.55357	$< 1 \times 10^{-6}$	0.061765	0.429757	$< 1 \times 10^{-6}$	0.276164
ANTI_APOPTOSIS	-0.57571	$< 1 \times 10^{-6}$	0.041176	0.273994	0.716216	0.896547
REGULATION_OF_APOPTOSIS	-0.47398	$< 1 \times 10^{-6}$	0.030882	0.323266	0.143868	0.441078
INDUCTION_OF_APOPTOSIS_BY_INTRACELLULAR_SIGNALS	-0.57402	$< 1 \times 10^{-6}$	0.024706	0.467932	0.241265	0.447509
DEBIASI_APOPTOSIS_BY_REOVIRUS_INFECTION_UP	-0.55115	$< 1 \times 10^{-6}$	0.020588	0.291691	0.442308	0.706462
HAMAI_APOPTOSIS_VIA_TRAIL_DN	-0.54161	$< 1 \times 10^{-6}$	0.033437	0.280911	0.695279	0.952039
LAU_APOPTOSIS_CDKN2A_UP	-0.47528	$< 1 \times 10^{-6}$	0.04059	0.275493	0.809942	0.862365

	$PKC\varepsilon^+ > PKC\varepsilon^-$			$PKC\delta^+ > PKC\delta^-$		
Survival-related gene sets	**ES**	**p-value**	**FDR**	**ES**	**p-value**	**FDR**
REGULATION_OF_MITOTIC_CELL_CYCLE	0.572949	0.033058	0.160091	-0.64041	0.019432	0.122893
SCIAN_CELL_CYCLE_TARGETS_OF_TP53_AND_TP73_UP	0.611301	0.097173	0.212901	-0.66298	0.111667	0.220461
GEORGES_CELL_CYCLE_MIR192_TARGETS	0.434973	0.058405	0.197834	-0.48895	0.055276	0.205917
REGULATION_OF_CELL_GROWTH	0.394842	0.287284	0.287284	-0.52386	0.095105	0.095105
SWEET_KRAS_ONCOGENIC_SIGNATURE	0.495281	0.01875	0.03175	-0.55626	0.019894	0.012733
CHIARADONNA_NEOPLASTIC_TRANSFORMATION_KRAS_CDC25_UP	0.541771	0.015798	0.009219	-0.75039	$< 1 \times 10^{-6}$	$< 1 \times 10^{-6}$
FIRESTEIN_PROLIFERATION	0.386646	0.062583	0.364772	-0.46776	0.029308	0.097521
ST_GRANULE_CELL_SURVIVAL_PATHWAY	0.368933	0.447167	0.451163	0.43769	0.347305	0.355097

single-gene analysis of our microarray expression data, focusing our subsequent analysis on PMA-up-regulated genes. PKC isozyme-regulated genes were defined as those in which induction by PMA was modified (either reduced or increased) by >50% by the corresponding RNAi depletion. Our single-gene analysis established unique as well as overlapping roles for PKCs in gene expression. A list of the top 100 PKC isozyme-regulated genes is presented in supplemental Table S5. Notably, PKCδ was the most relevant isozyme implicated in up-regulation of genes by PMA. Overall, the relative contribution of each PKC isozyme to the PMA response was as follows: PKCδ > PKCε > PKCα (73, 40, and 23%, respectively) (Fig. 2*C*). PKC isozyme-regulated genes were classified according to two different patterns: Pattern A, in which RNAi reduced PMA induction by >50%, and Pattern B, in which RNAi augmented PMA induction by >50% (examples shown in Fig. 2*D*). Most of the PKCδ-regulated genes belong to Pattern A, an indication that this PKC functions largely as a positive regulator of PMA-induced transcription (Fig. 2*E*).

A more stringent analysis in which specificity was defined as >50% change in PMA-mediated induction upon depletion of one PKC isozyme with <25% change upon depletion of the other two PKCs identified 75 specific PKCδ-regulated genes, 57 (76%) of which followed Pattern A. On the other hand, PKCα and PKCε had negligible specificity on gene expression (0 and 4 specific genes, respectively). A list of PKC isozyme-specific regulated genes is presented in Table 2. Altogether, these results argue for a prominent role for PKCδ as a mediator of gene induction by phorbol esters.

Differential Contribution of PKC Isozymes to Basal Expression of Genes; a Key Role for PKCε—Next, we examined the relative contribution of PKC isozymes to gene expression in cells growing in normal medium (with 10% FBS). The involvement of individual PKCs to basal gene expression was strikingly different than that observed for PMA-regulated genes (PKCε > PKCδ > PKCα; 67, 37, and 32%, respectively). 41% of the genes were regulated by PKCε, compared with 19% by PKCδ and 11% by PKCα (Fig. 2*F*). Thus, whereas PKCδ mainly regulates the PMA response, PKCε controls gene expression in a "physiological" setting. The overall contribution of PKCα to both basal and PMA-induced gene expression is comparatively lesser.

We classified these genes into two patterns: those in which basal expression was either reduced (Pattern A) or increased (Pattern B) by >50% by the corresponding RNAi depletion (examples shown in Fig. 2*G*). RNAi depletion for any of the three PKCs led mostly to enhanced basal gene expression (Pattern B), suggestive of a negative role for PKCs in transcriptional regulation under growth factor-stimulated conditions. The effect was very prominent for the PKCε-regulated genes (Fig. 2*H*), suggesting that PKCε generally regulates gene expression in a negative manner.

Antagonistic Regulation of Apoptotic and Survival Gene Sets by PKCδ and PKCε—An issue that remained unexplored to date is whether the opposite regulation of cell death by PKCδ and PKCε can be explained by differential regulation of proapo-

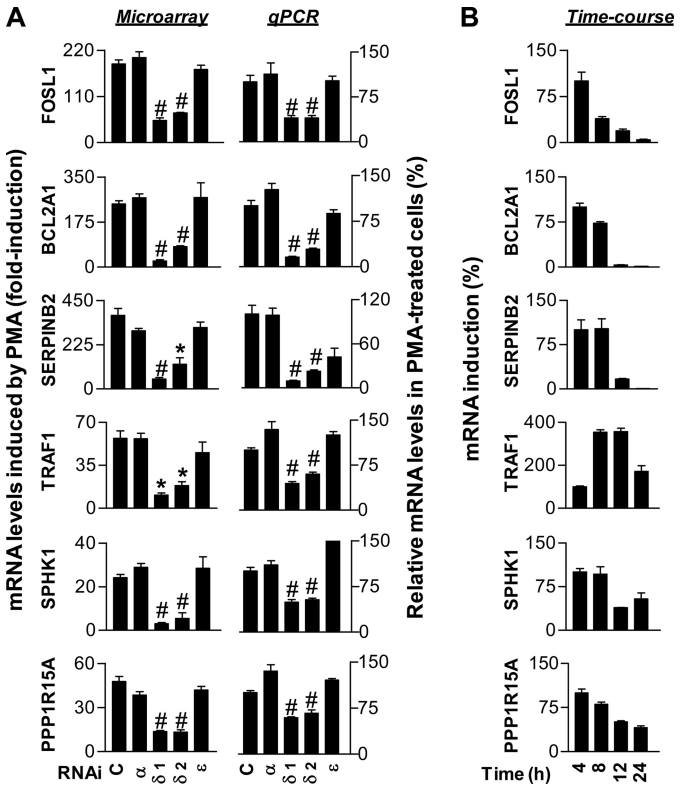


FIGURE 3. **Identification of novel PKCδ target genes.** *A*, LNCaP cells were transfected with RNAi duplexes for PKCs (*α1*, *δ1*, *δ2*, and *ϵ1*) or control (*C*), and mRNA expression of PKCδ-regulated genes (*FOSL1*, *BCL2A1*, *SERPINB2*, *TRAF1*, *SPHK1*, or *PPP1R15A*) in response to PMA (100 nM, 1 h) was determined. *Left*, mRNA levels from the microarray analysis. *Right*, validation of mRNA levels using qPCR. *B*, time course expression analysis of PKCδ-specific genes. The relative mRNA levels were calculated relative to expression at 4 h. Data are expressed as mean ± S.E. (*error bars*) ($n = 3$). *, $p < 0.01$; #, $p < 0.001$.

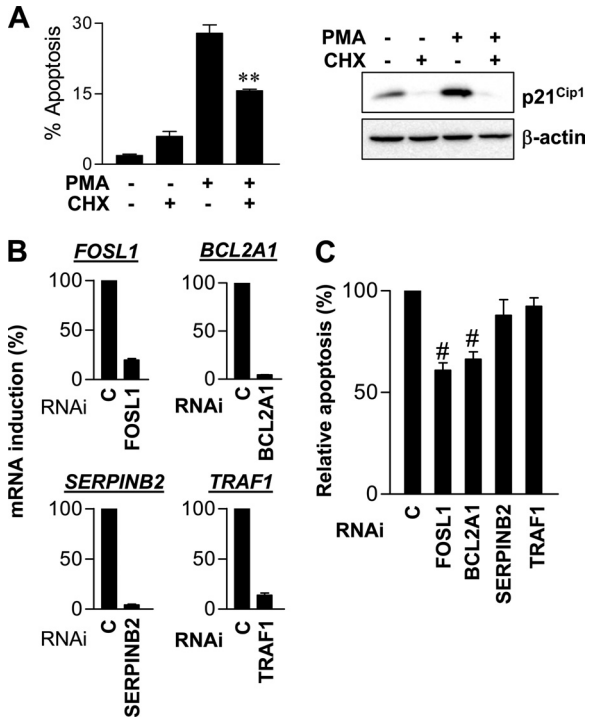


FIGURE 4. **PMA-induced apoptosis requires PKCδ-mediated induction of *FOSL1* and *BCL2A1*.** *A*, LNCaP cells were pretreated with cycloheximide (*CHX*; 50 μM, 50 min) followed by PMA treatment (100 nM, 1 h). *Left*, the incidence of apoptosis was assessed 24 h later. *Right*, as a positive control, we measured the induction of $p21^{cip1}$ by Western blot 4 h post-treatment, which is blocked by CHX. A representative Western blot is shown ($n = 3$). *B* and *C*, LNCaP cells transfected with RNAi duplexes for *FOSL1*, *BCL2A1*, *SERPINB2*, or *TRAF1* were treated with PMA. *B*, mRNA levels were measured 5 h later by qPCR. *C*, the incidence of apoptosis was determined 24 h later. In all cases, data are presented as mean ± S.E. (*error bars*) ($n = 3$). **, $p < 0.01$; #, $p < 0.001$.

ptotic *versus* prosurvival pathways. Analysis of gene expression profiles comparing PKCδ and PKCϵ contribution at the single-gene level rendered only few genes regulated in an opposite manner by these PKCs (supplemental Fig. S2).

In order to investigate global gene expression changes related to apoptosis, we undertook a computational approach. A collection of apoptosis-related gene sets was derived from publicly available catalogs at MSigDB (see "Experimental Procedures" and supplemental Table S1). GSEA results for PKC isozyme-expressing *versus* PKC isozyme-depleted cells ($PKC^+ > PKC^-$) are shown in Table 3 (top). We found that eight apoptosis-related gene sets were regulated by PKCδ with $p < 0.05$ and FDR < 0.25. On the other hand, none of these gene sets were enriched in PKCϵ-expressing samples (none is both $p < 0.05$ and FDR < 0.25). In order to obtain the common core apoptotic genes regulated by PKCδ, we ran a post-GSEA leading edge analysis based on the high scoring gene sets (supplemental Table S6). Importantly, some of the core apoptotic genes identified with this approach were also previously found as PKCδ-specific genes in our single-gene analysis, such as *BCL2A1*, *SPHK1* (sphingosine kinase 1), and *PPP1R15A* (protein phosphatase 1, regulatory subunit 15A).

To determine whether PKCϵ regulates prosurvival/proliferative pathways, we defined a collection of survival-related gene sets from MSigDB. We were able to identify three gene sets that positively correlate with PKCϵ ($p < 0.05$ and FDR < 0.25; Table 3, bottom), which include gene sets for mitotic cell cycle regulation, K-Ras-mediated oncogenesis, and transformation. Notably, these gene sets were in all cases inversely correlated with PKCδ with $p < 0.05$ and FDR < 0.25. Therefore, PKCδ and PKCϵ regulate antagonistic subsets of genes in the context of apoptosis and survival.

Identification of Novel Genes Implicated in PKCδ-mediated Apoptosis—A subset of genes specifically regulated by PKCδ (*FOSL1*, *BCL2A1*, *TRAF1*, *SERPINB2*, *SPHK1*, and *PPP1R15A*) was selected for validation of the microarray results using qPCR. There was a remarkable agreement between microarray data and qPCR analysis for all selected genes, and in all cases, PKCδ RNAi, but not PKCα or PKCϵ RNAi, markedly inhibited their induction by PMA (Fig. 3*A*, compare *left* and *right panels*). Time course expression analysis of these genes showed that they were in all cases early regulated genes (Fig. 3*B*).

We have previously established that phorbol esters trigger a pronounced apoptotic response in androgen-dependent prostate cancer cells, an effect primarily mediated by PKCδ (6, 10). Notably, the apoptotic effect of PMA in LNCaP cells was reduced approximately by half by the protein synthesis inhibitor cycloheximide (Fig. 4*A*). Thus, it is conceivable that transcriptional mechanisms mediate, at least partially, the apoptotic response induced by PKCδ activation.

In order to determine the potential involvement of candidate genes in this effect, we conducted selective RNAi-mediated

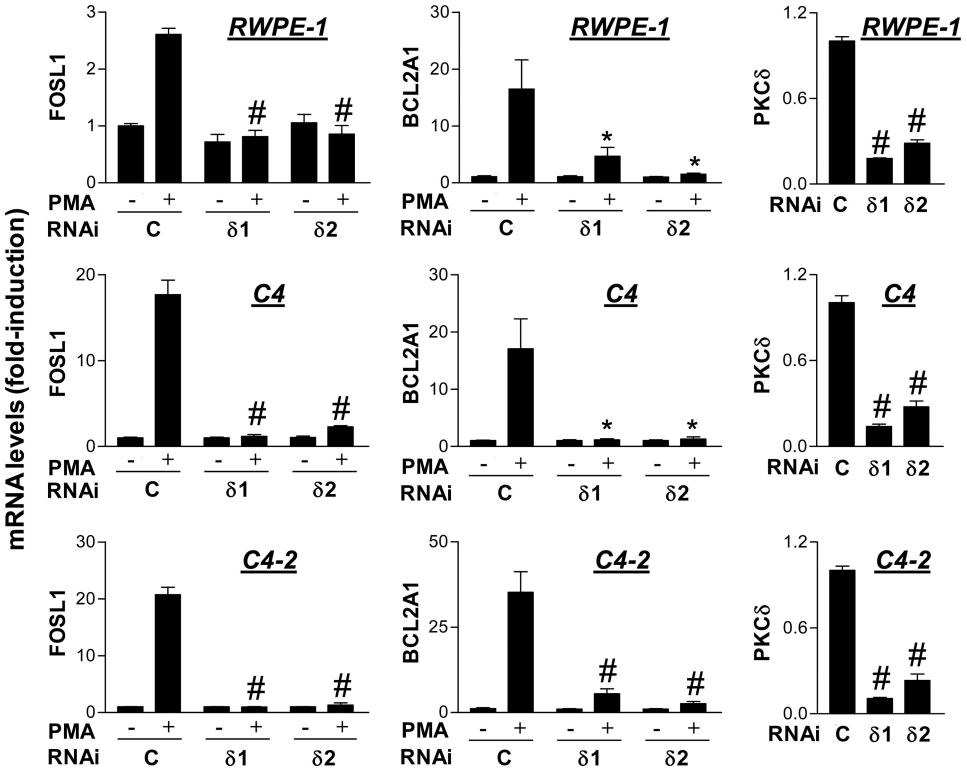


FIGURE 5. **Validation of *FOSL1* and *BCL2A2* as PKCδ targets in prostate cell lines.** RWPE-1 immortalized prostate epithelial cells or prostate cancer cell lines C4 and C4-2 were transfected with RNAi duplexes for PKCδ (*δ1* and *δ2*) or control (*C*). mRNA levels for *FOSL1 (left panels*) and *BCL2A1 (middle panels*) in response to PMA (100 nM, 1 h) were determined by triplicate by qPCR. Depletion efficiency for PKCδ was determined by qPCR (*right panels*). Data are expressed as mean ± S.E. (*error bars*) ($n = 3$). *, $p < 0.01$; #, $p < 0.001$.

knockdown for four different PKCδ-regulated genes (*BCL2A1*, *FOSL1*, *TRAF1*, or *SERPINB2).* Transfection of specific siRNA duplexes for each gene into LNCaP cells markedly reduced their induction by PMA (Fig. 4*B*). Remarkably, knockdown of either *BCL2A1* or *FOSL1* significantly reduced the apoptotic effect of PMA in LNCaP cells to a similar extent as observed with CHX. On the other hand, depletion of *SERPINB2* or *TRAF1* did not have a significant effect (Fig. 4*C*). Additional experiments using two separate RNAi duplexes for *FOSL1* and *BCL2A1* gave similar results (supplemental Fig. S3).

Next, we determined whether our findings also applied to other prostate cell lines. We found that PMA induces *FOSL1* and *BCL2A1* in C4 and C4-2 prostate cancer cells as well as in non-transformed immortalized RWPE-1 cells. Furthermore, as in LNCaP cells, the induction of these genes by PMA is markedly reduced in PKCδ-depleted C4, C4-2, and RWPE-1 cells (Fig. 5). In conclusion, our findings are of general relevance to different prostate cell lines.

To further extend the relevance of our findings, we evaluated prostate cancer data sets available through the Oncomine repository (available on the World Wide Web). Interestingly, the PKCδ-regulated genes *FOSL1*, *BCL2A1*, *TRAF1*, *SERPINB2*, *SPHK1*, and *PPP1R15A* were down-regulated in human prostate cancer compared with normal prostate, as revealed in multiple data sets (Fig. 6*A*, *left*). Comparison of available data sets by meta-analysis showed statistically significant differences for each PKCδ-regulated gene (Fig. 6*A*, *right*). Representative examples for each gene are shown in Fig. 6*B*. Even more, all of these genes were included in the top 10% underexpressed genes associated with the gene expression signature for prostate cancer ($p < 0.05$; Fig. 6*C*). Interestingly, we found a significant association between androgen independence or Gleason scores and down-regulation of PKCδ target genes (Fig. 6*D*).

Etoposide-induced Apoptosis in LNCaP Cells Is Mediated by PKCδ-regulated Genes FOSL1 and BCL2A1—It has been established that chemotherapeutic agents require PKCδ for their cell killing effect, including in prostate cancer cells (26–29). In that regard, etoposide partially depends on PKCδ to promote apoptotic death in LNCaP cells because PKCδ depletion with two separate siRNA duplexes reduced its apoptotic effect by ~40% (Fig. 7, *A* and *B*). Etoposide treatment caused increased levels of *BCL2A1*, *FOSL1*, *SERPINB2*, and *TRAF1* mRNA in LNCaP cells (Fig. 7*C*), which were partially sensitive to PKCδ RNAi depletion (Fig. 7*D*).

We speculated that genes up-regulated in response to PKCδ activation are possibly implicated in the apoptotic effect of etoposide. To test this hypothesis, we used LNCaP cells subject to RNAi depletion for PKCδ-regulated genes (*FOSL1*, *BCL2A1*, *SERPINB2*, or *TRAF1*). Up-regulation of mRNA levels for each of these genes by etoposide was impaired in cells transfected with each corresponding RNAi duplex (Fig. 8*A*). Knockdown of *FOSL1* and *BCL2A1* reduced significantly the apoptotic effect of etoposide in LNCaP cells (Fig. 8*B*). Thus, PKCδ relies on a common set of genes to induce apoptosis in response to diverse stimuli.

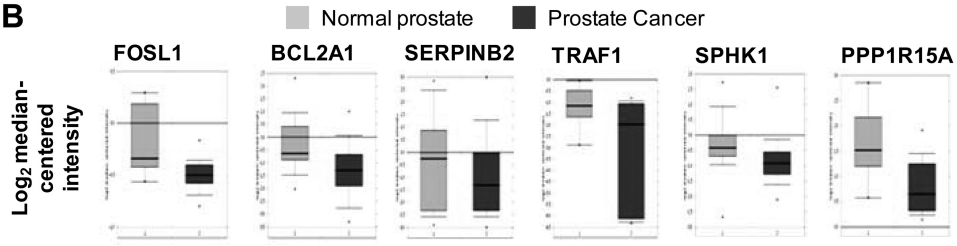
DISCUSSION

Here, we report the first genome-wide analysis of transcriptional regulation by PKC isozymes. A longitudinal analysis of

A

Analysis type: Prostate Cancer vs. Normal						Meta-analysis		
Gene	p value	fold PC/N	PC (n)	N (n)	Dataset	Gene	Median rank	p value
	0.004	-1.632	**3**	**3**	Tomlins			
	0.022	-1.359	52	50	Singh			
FOSL1	0.027	-1.199	27	8	Vanaja	**FOSL1**	1183	0.022
	9.38×10^{-4}	-1.818	69	20	Wallace			
BCL2A1	0.038	-1.08	27	8	Vanaja	**BCL2A1**	2191	0.019
SERPINB2	0.032	-1.511	52	50	Singh	**SERPINB2**	only one Study	
	0.002	-3.229	25	9	Welsh			
TRAF1	0.029	-1.716	52	50	Singh	**TRAF1**	1002.5	0.015
	0.005	-18.84	15	15	Luo 2			
	0.011	-1.421	69	20	Wallace			
SPHK1	0.047	-1.111	27	8	Vanaja	**SPHK1**	2898	0.047
	0.002	-1.454	15	15	Luo 2			
PPP1R15A	0.017	-1.669	27	8	Vanaja	**PPP1R15A**	884.5	0.009

B



C

Oncomine's Primary Concept: Gene Expression Signatures for Prostate Cancer		
Gene	**Associated Concept**	**Study**
FOSL1	Top 5% Under-expressed	Tomlins
	Top 1% Under-expressed	Luo 2
BCL2A1	Top 10% Under-expressed	Vanaja
SERPINB2	no data	
TRAF1	Top 10% Under-expressed	Welsh
SPHK1	Top 5% Under-expressed	Luo 2
	Top 1% Under-expressed	Luo 2
PPP1R15A	Top 10% Under-expressed	Vanaja

D

	Prostate cancer Downregulated Genes			
	Androgen-ablation resistance		Gleason score	
Gene	**p-value**	**FDR**	**p-value**	**FDR**
FOSL1	8.88×10^{-4}	0.205	2.42×10^{-7}	1.33×10^{-4}
BCL2A1	7.90×10^{-4}	0.188	0.003	0.213
SERPINB2	7.9×10^{-4}	0.188	0.003	0.213
TRAF1	4.38×10^{-4}	0.14	no data	
SPHK1	6.54×10^{-4}	0.044	2.85×10^{-25}	2.65×10^{-21}
PPP1R15A	9.01×10^{-6}	0.014	2.85×10^{-25}	2.65×10^{-21}

FIGURE 6. **Expression of PKCδ-regulated genes in human prostate tissues.** Publicly available data sets of human prostate tissues from Oncomine were analyzed as described under "Experimental Procedures." *A*, *left*, -fold change and statistics for the expression of PKCδ-regulated genes in studies available in Oncomine. *Right*, a comparison between studies for each gene is provided as meta-analysis. *PC*, prostate cancer; *N*, normal prostate. *B*, representative box plots for PKCδ-regulated genes were derived from the following studies: Vanaja prostate (*FOSL1*, *PPP1R15A*), Wallace prostate (*BCL2A1*), Singh prostate (*SERPINB2*), and Welsh prostate (*TRAF1*). *C*, significant association between PKCδ-regulated genes and the Oncomine "Gene Expression Signature for Prostate Cancer." *D*, association significance for androgen ablation-resistant prostate tumors and Gleason scores with down-regulation of PKCδ target genes.

the LNCaP cell transcription profile over 24 h after a short exposure to the phorbol ester PMA revealed distinct patterns of gene expression. Major changes occur at early times; however, a large number of genes become either up- or down-regulated at later stages, possibly involving secondary loops of activation by transcription factors induced by PMA at early times. Secondary transcriptional events may also explain the sustained expression of many early genes.

As anticipated from their distinctive functional properties, PKC isozymes exhibit both overlapping and selective roles in the control of gene expression. We identified PKCδ as the major mediator of gene induction by PMA. A sizeable number of genes were regulated specifically by PKCδ without significant contribution of the other phorbol ester-sensitive PKCs. Our previous studies found that PKCδ-mediated apoptosis in LNCaP cells involves a dual regulation; on one hand, PKCδ promotes the release of death factors, primarily TNFα, and on the other hand, it mediates apoptosis by activation of death receptors (6). The present study indicates that PKCδ also controls the expression of components of the death receptor cascade, including TNFAIP2 (TNFα-induced protein 2); TNF receptor members 11a, 11b, and 12; and TRAF1 (TNF receptor-associated factor 1), suggesting that a significant component of the TNFα response upon phorbol ester stimulation may be mediated by transcriptional mechanisms.

Our studies identified *FOSL1*, *BCL2A1*, *SERPINB2*, and *TRAF1* as PKCδ-regulated genes. These genes were chosen not only for their relevance in apoptotic, survival, and/or mitogenic signaling (30–33) but also because they are in all cases induced by TNFα (34–37). *BCL2A1* encodes a member of the BCL-2 protein family and is up-regulated by phorbol esters and inflammatory cytokines. *FOSL1* belongs to the Fos gene family that consists of four members (*FOS*, *FOSB*, *FOSL1*, and *FOSL2*). Proteins encoded by these genes are components of the transcription factor complex AP-1 and have been widely implicated in cell proliferation, differentiation, and transformation (38, 39). Although antiapoptotic functions for *BCL2A1* and *FOSL1* have been established, a recent study found that *FOSL1* is associated with sensitivity to cell death by the epidermal growth factor receptor inhibitor erlotinib in glioblastoma (40). A pro-apoptotic role for *BCL2A1* has also been described (31, 41). We could not find any involvement of *SERPINB2* and *TRAF1* in

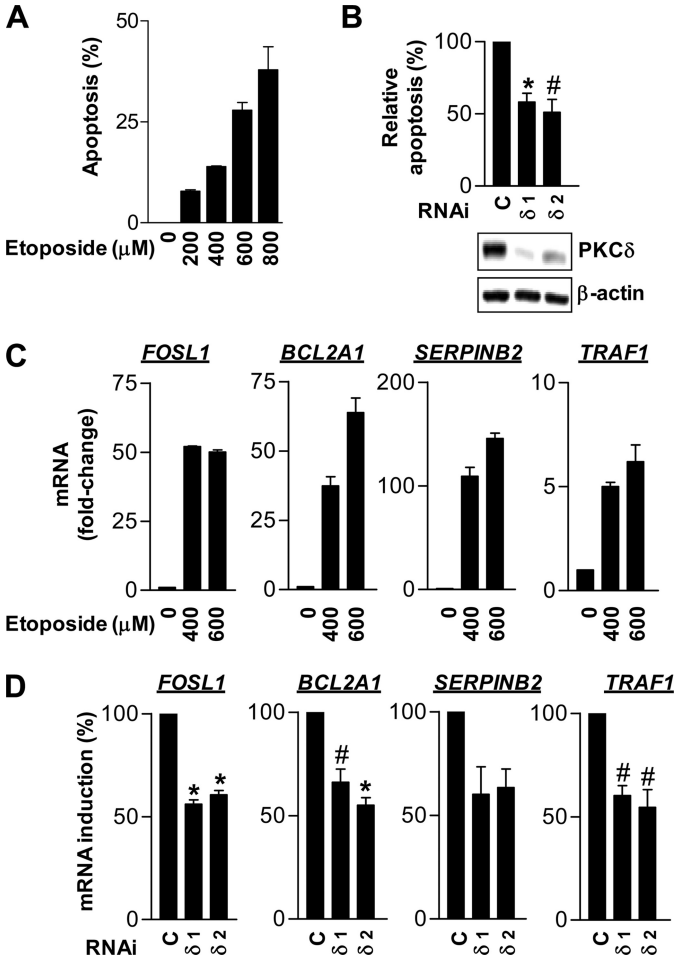


FIGURE 7. **Etoposide promotes cell death and induces the expression of PKCδ-regulated genes.** LNCaP cells were serum-starved for 48 h and then treated with increasing concentrations of etoposide (200–800 μM). Where indicated, cells were transfected with RNAi duplexes for PKCδ (*δ1* or *δ2*) or control (*C*) prior to the addition of etoposide. *A* and *B*, the incidence of apoptosis was assessed 24 h after the addition of etoposide. A representative Western blot showing the depletion of PKCδ is shown ($n = 3$). *C* and *D*, mRNA levels were determined by qPCR 5 h later. In all cases, data are represented as the mean ± S.E. (*error bars*) ($n = 3$). #, $p < 0.05$; *, $p < 0.01$.

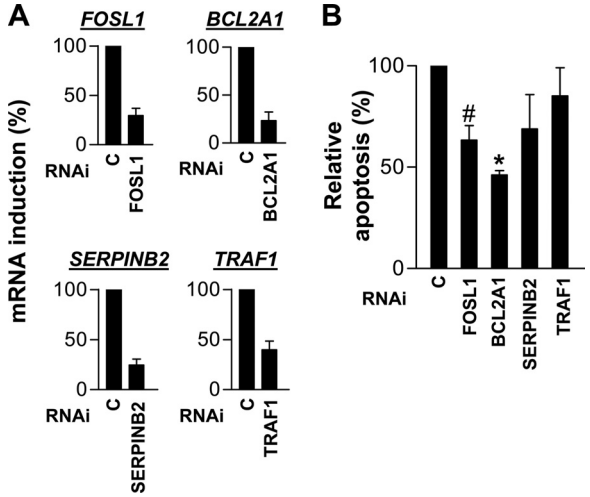


FIGURE 8. **PKCδ-regulated genes mediate cell death by etoposide.** LNCaP cells were transfected with RNAi duplexes for *FOSL1*, *BCL2A1*, *SERPINB2*, or *TRAF1*, serum-starved for 48 h, and treated with etoposide (600 μM). *A*, mRNA levels for PKCδ-regulated genes in response to etoposide were determined and expressed as percentage of control. *B*, apoptosis was determined 24 h later. Data are represented as the mean ± S.E. (*error bars*) ($n = 3$). #, $p < 0.05$; *, $p < 0.01$.

PMA- and etoposide-induced apoptosis despite the fact that it is up-regulated in a PKCδ-dependent manner, suggesting that these genes may be implicated in other functions driven by PKCδ activation. We cannot rule out a potential contribution of *SERPINB2* and *TRAF1* in response to other apoptotic stimuli. The requirement of *FOSL1* and *BCL2A1* for the apoptotic effect of PMA and etoposide points to the multifocal nature of PKCδ targets and highlights the contribution of transcriptional mechanisms to apoptosis mediated by this kinase. In this regard, it is important to mention that PKC isozymes can also regulate post-transcriptional mechanisms, including mRNA stability and degradation (42), which may account for some of the changes observed in our microarray study. Likewise, the implication of PKCs, including PKCδ, in post-translational control is well established (43), suggesting multiple mechanisms by which PKC isozymes could ultimately regulate protein expression.

Although it is generally accepted that phorbol esters mimic DAG effects in cells, physiological and pharmacological activation of PKCs are not necessarily equivalent. PMA causes sustained translocation and activation of PKCs compared with DAG, a short lived lipid second messenger that is transiently generated in response to receptor activation (44). Notably, diverse stimuli cause redistribution of PKC isozymes to different intracellular compartments, leading to their differential access to substrates and ultimately to unique signaling inputs (1). Therefore, divergent patterns in the kinetics of activation and intracellular relocalization of PKC isozymes may account for the differences in gene regulation under basal (serum) and PMA-stimulated conditions. Interestingly, PKCs have a prominent role in repressing basal (serum-stimulated) gene expression because RNAi depletion of any of the PKCs leads predominantly to the up-regulation of genes. However, it has to be taken into consideration that the potential role of PKCs in maintaining basal gene expression may be underestimated due to the inherent limitations in microarray sensitivity for detecting changes in gene repression, particularly for genes with low basal expression. Nevertheless, it is striking that under serum stimulation, PKCε plays such an important role relative to the other PKCs, arguing that PKCε may be an important mediator of the transcriptional effects of growth factors present in the serum (45–47). Based on our microarray data, PKCε acts primarily as a repressor of basal and phorbol ester-dependent gene expression. Despite the well established "Yin-Yang" relationship described for PKCδ and PKCε in signaling (1), particularly in prostate cancer (6, 10, 12), we found only few genes regulated by these PKCs in an opposite manner (such as *RASSF9*, *BCL2L14*, *EGR1*, and *CAV1*). It would be interesting to further study these genes in the context of apoptosis and survival. Notably, GSEA revealed that PKCδ controls proapoptotic gene sets, whereas PKCε regulates antiapoptotic gene sets. We speculate that the antagonistic regulation of apoptotic genes by PKCδ and PKCε may contribute to their contrasting responses. Notably, analysis of transcriptional networks revealed unique elements in the promoters regulated by PKCδ and PKCε. For example, Sp1, AP2, and CREB binding elements are overrepre-

sented in the promoters of PKCδ-regulated genes, whereas AP1, NF-κB, and SRE sites are frequently present in PKCε-regulated gene promoters.[4]

In summary, this study represents the first comprehensive evaluation of genome-wide transcription dynamics in response to the activation of PKC isozymes. We were able to unearth patterns of specific gene expression regulated by individual members of the PKC family and identify novel genes that mediate PKCδ-driven apoptosis. PKC modulators, such as bryostatins, ingenol derivatives, and phorbol esters, are in clinical trials for different types of malignancies (1, 48); hence, the identification of genes that modulate the resistance of cancer cells to these agents or other drugs acting through PKCs may have significant therapeutic implications.

Acknowledgments—We thank Dr. John Tobias and Dr. Don Baldwin for invaluable help with microarray data analysis and interpretation.

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[4] M. C. Caino and M. G. Kazanietz, unpublished observations.